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GENOME REPLICATION AND REGULATION OF VIRAL GENE EXPRESSION

Genetic Variability of Long Terminal Repeat Region between HIV-2 Groups Impacts Transcriptional Activity

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ABSTRACT The HIV-2 long terminal repeat (LTR) region contains several transcription factor (TF) binding sites. Efficient LTR transactivation by cellular TF and viral proteins is crucial for HIV-2 reactivation and viral production. Proviral LTRs from 66 antiretroviral-naive HIV-2-infected patients included in the French ANRS HIV-2 CO5 Cohort were sequenced. High genetic variability within the HIV-2 LTR was observed, notably in the U3 subregion, the subregion encompassing most known TF binding sites. Genetic variability was significantly higher in HIV-2 group B than in group A viruses. Notably, all group B viruses lacked the peri-ETS binding site, and 4 group B sequences (11%) also presented a complete deletion of the first Sp1 binding site. The lack of a peri-ETS binding site was responsible for lower transcriptional activity in activated T lymphocytes, while deletion of the first Sp1 binding site lowered basal or Tat-mediated transcriptional activities, depending on the cell line. Interestingly, the HIV-2 cellular reservoir was less frequently quantifiable in patients infected by group B viruses and, when quantifiable, the reservoirs were significantly smaller than in patients infected by group A viruses. Our findings suggest that mutations observed *in vivo* in HIV-2 LTR sequences are associated with differences in transcriptional activity and may explain the small cellular reservoirs in patients infected by HIV-2 group B, providing new insight into the reduced pathogenicity of HIV-2 infection.

IMPORTANCE Over 1 million patients are infected with HIV-2, which is often described as an attenuated retroviral infection. Patients frequently have undetectable viremia and evolve at more slowly toward AIDS than HIV-1-infected patients. Several studies have reported a smaller viral reservoir in peripheral blood mononuclear cells in HIV-2-infected patients than in HIV-1-infected patients, while others have found similar sizes of reservoirs but a reduced amount of cell-associated RNA, suggesting a block in HIV-2 transcription. Recent studies have found associations between mutations within the HIV-1 LTR and reduced transcriptional activities. Until now, mutations within the HIV-2 LTR region have scarcely been studied. We conducted this research to discover if such mutations exist in the HIV-2 LTR and their potential association with the viral reservoir and transcriptional activity. Our study indicates that transcription of HIV-2 group B proviruses may be impaired, which might explain the small viral reservoir observed in patients.

KEYWORDS long terminal repeat, ETS transcription factors, human immunodeficiency virus, transcription factors

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The AIDS epidemic is due to two different lentiviruses: human immunodeficiency virus type 1 (HIV-1) and HIV-2 (1, 2). HIV-1 is responsible for more than 99% of all HIV infections. HIV-2 infections are mainly encountered in West African countries and in countries with historical and economic links with those countries, notably, Portugal and France (3, 4). The HIV-1 and HIV-2 genomes differ at the nucleotide level by 40% to 60%, depending on the genomic region (5). HIV-2 is often considered an attenuated model of HIV infection. Indeed, patients infected with HIV-2 progress more slowly toward AIDS (6, 7) and present a slower decline of the CD4 cell count (8). HIV-2 plasma viral loads (VL) are frequently undetectable in the absence of antiretroviral (ARV) treatment (9, 10), and transmission rates are lower than for HIV-1, either by sexual (11) or maternal (12) transmission. HIV-2 is divided into 9 groups, but only groups A and B have spread (4, 13). Over 90% of worldwide HIV-2 infections are caused by group A viruses, while group B viruses account for less than 10% of HIV-2 infections (14). HIV-2 groups A and B differ in their geographical distributions: group A has spread from Guinea-Bissau to other West African countries, while group B is mainly localized in Ivory Coast, Ghana, and Burkina Faso (3, 14).

Previous studies have assessed the HIV-2 DNA reservoir in peripheral blood mononuclear cells (PBMC) in West African countries and reported sizes of cellular reservoirs similar to those of HIV-1 but lower levels of cell-associated RNA (8, 15–17). High HIV-2 DNA levels were correlated strongly with high HIV-2 RNA levels and low CD4 cell counts (8, 15, 16). Smaller cellular HIV-2 reservoirs were described in other studies that included patients with undetectable HIV-2 plasma VL and higher CD4 cell counts (9, 18).

The long terminal repeat (LTR) region plays a major role in HIV-2 transcription and reactivation. The LTR is subdivided into 3 regions: U3, R, and U5. LTR transcriptional activity is enhanced by different cellular transcription factors (TFs), including Elf-1, Sp1, and NF-κB (19–21), and by the viral protein Tat (22). A limited subregion of the HIV-2 LTR, here named the "regulatory" subregion, encompasses all known cellular TF binding sites (23, 24).

Fewer transcription factor binding sites have been described in HIV-2 than in HIV-1, and two main differences are observed. While HIV-1 usually exhibits two NF-κB binding sites that are sufficient for response to cellular activation, only one NF-κB binding site is present in the HIV-2 LTR, and to efficiently respond to cellular activation, HIV-2 requires the presence of four *cis*-acting synergistic subelements: two purine-rich binding sites, purine box 1 (PuB1) and PuB2, that bind proteins of the ETS family, like Elf-1; a peri-ETS (pETS) binding site located between PuB1 and PuB2 that also binds Elf-1; and a monocyte-specific peri-κB sequence (25, 26).

The expression levels of some TFs are modulated by the cellular activation status, such as upregulation of Elf-1 in activated T cells (26). The three Sp1 binding sites have been linked to the basal activity of the LTR promoter and to the response to transactivation by Tat (27).

HIV-2 LTR genetic diversity, especially in TF binding sites, may alter its ability to be efficiently activated by cellular TFs and/or by Tat (28, 29). It has been demonstrated for HIV-1 that variation in the number of NF-κB binding sites altered the level of *in vitro* replication (30). Variability within HIV-2 LTR sequences could also impact the reactivation of HIV-2 proviruses, as recently shown for HIV-1 (29). Until now, only two studies have been conducted on HIV-2 LTRs, and both focused on group A viruses, due to the low number of group B infections worldwide (21, 24). However, in the French HIV-2 ANRS CO5 Cohort, group B infections are more frequent, representing around 30% of HIV-2-infected individuals (31). Furthermore, in our cohort, the first data on the HIV-2 DNA level showed that patients exhibited smaller HIV-2 cellular reservoirs (32, 33) than in previous studies (8, 15, 16). We wondered if the HIV-2 cellular reservoir could be linked to LTR transcriptional activity and if genetic diversity within the LTR had an impact on its transcriptional activity.

The aim of this study was to assess genetic diversity within the HIV-2 LTR, as well as the transcriptional activity of the HIV-2 LTR, and their potential association with the size

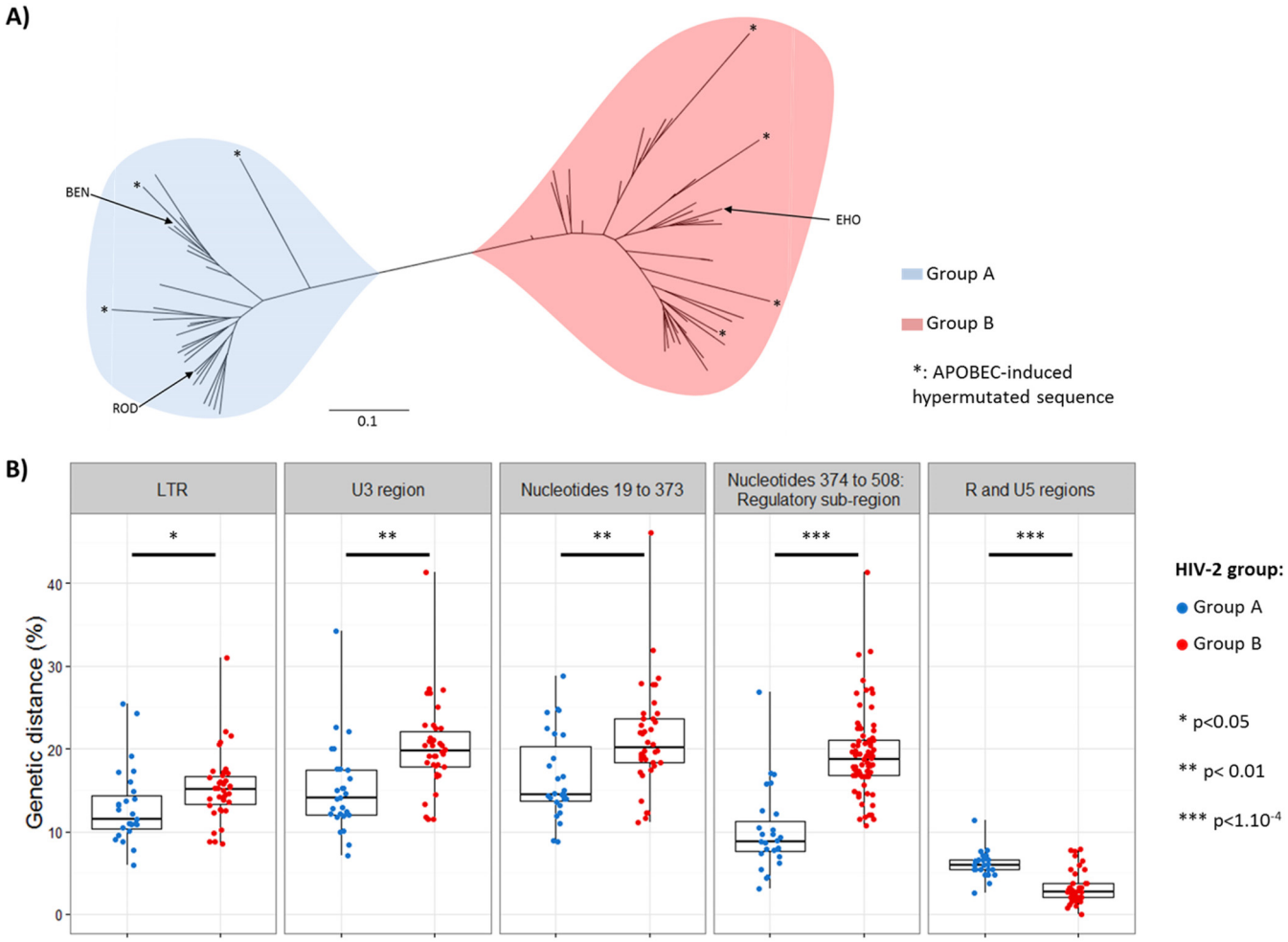

FIG 1 Phylogenetic tree and genetic distances between HIV-2 LTR sequences. (A) Phylogenetic tree obtained with LTR sequences from 65 patients included in this study and 3 HIV-2 reference strains (ROD, BEN, and EHO for subtypes A1 and A2 and group B, respectively). As recommended in phylogenetic analysis, the recombinant A/B sequence was excluded. The phylogenetic tree was generated with PhyML using a general time reversible (GTR) model with gamma distribution at 4 and 1,000 bootstraps and then edited on FigTree. The lengths of branches are proportional to genetic distances; the length of the scale depicted in the figure is equal to 0.10 substitution per nucleotide. (B) Genetic distances in the whole HIV-2 LTR and in different subregions. The genetic distances of group A and B viruses were obtained by comparison with their reference strains, ROD and EHO, respectively. Those genetic distances are plotted separately, with each circle corresponding to one patient. The box plots represent the medians and interquartile ranges of genetic distances; *P* values were calculated with a Mann-Whitney test.

of the HIV-2 cellular reservoir in a population of antiretroviral-naive HIV-2-infected patients.

RESULTS

Patients' characteristics. The LTR region was sequenced in 66 patients out of 102 (65%) with available PBMC. Phylogenetic analysis showed the following distribution among HIV-2 groups: 27 A (41%), 38 B (58%), and 1 A/B recombinant (1%) (Fig. 1A). In group A, 19 sequences belonged to subtype A1 and 8 to subtype A2. We combined subtype A1 and A2 viruses in our analyses, as their respective numbers were too small. The A/B recombinant clustered with group B sequences for nucleotides 120 to 320 and with group A sequences for the rest of its LTR. Patients infected by HIV-2 group A and B viruses did not differ in their clinical, biological, and immunological characteristics, as depicted in Table 1. Only 9 patients (14%) had RNA plasma VL above 40 copies/ml (c/ml): 4 were infected with a group A virus (median = 68 c/ml), 4 with a group B virus (median = 78 c/ml), and 1 with the recombinant virus (VL = 829 c/ml) (Table 1).

Analysis of LTR genetic variability. The median LTR genetic distance was significantly lower in group A sequences (11.5%) than in group B sequences (15.2%; $P = 0.02$)

TABLE 1 Demographic, immunological, and biological characteristics of patients

Characteristic	Value for patients infected with HIV-2: All ($n = 66$)	Group A ($n = 27$)	Group B ($n = 38$)	A/B recombinant ($n = 1$)	*P* value (A vs B)[a]
Age (yr) [median (IQR)]	51 (47–55)	53 (47–56)	50 (46–54)	47	0.19
Women [*n* (%)]	45 (68)	17 (63)	28 (74)	0 (0)	0.42
West African origin [*n* (%)]	60 (91)	23 (85)	36 (95)	1 (100)	0.22
Heterosexual transmission [*n* (%)]	57 (86)	24 (89)	32 (84)	1 (100)	0.72
Time since HIV diagnosis (yr) [median (IQR)] ($n = 56$)	12 (8–17)	11 (5–17)	12 (9–17)	NA[b]	0.50
CDC clinical stage A [*n* (%)]	65 (98)	27 (100)	37 (97)	1 (100)	1
CD4 cell count/mm^3 [median (IQR)]	754 (540–882)	662 (485–892)	791 (581–878)	797	0.34
Patients with plasma VL of >40 c/ml [*n* (%)]	9 (14)	4 (15)	4 (11)	1 (100)	0.71
Median plasma VL (IQR) in patients with quantifiable viremia (c/ml)	80 (74–124)	68 (52–92)	78 (69–92)	829	0.71
Patients with quantifiable HIV-2 total DNA [*n* (%)]	24 (36)	18 (67)	6 (16)	0 (0)	$<1.10^{-4}$
Median cellular reservoir size (IQR) in patients with quantifiable HIV-2 total DNA ($\log_{10}$ c/10^6 PBMC)	2.04 (1.81–2.15)	2,11 (1.94–2.16)	1.75 (1.68–1.81)	NA	0.03
Patients with detectable but unquantifiable HIV-2 total DNA [*n* (%)]	40 (61)	8 (30)	31 (81)	1 (100)	$<1.10^{-4}$
Patients with undetectable HIV-2 total DNA [*n* (%)]	2 (3)	1 (3)	1 (3)	0 (0)	1

[a]Characteristics of group A- and group B-infected patients were compared using Fisher's exact tests and Mann-Whitney tests.
[b]NA, not applicable.

(Fig. 1B). Taken together, HIV-2 group A and B sequences showed a significantly higher median genetic distance in the U3 subregion than in the R-plus-U5 subregion (18.0% versus 4.8%; $P < 1.10^{-4}$). To compare this genetic diversity to that observed in the HIV-1 LTR, we determined the median genetic distance between the 1,194 HIV-1 group M sequences available in the Los Alamos HIV Compendium and the reference strain BRU. For the HIV-1 LTR, the median genetic distance was 17.3% (interquartile range [IQR] = 12.0 to 19.1%), with higher variability in the U3 subregion than in the R and U5 subregions (data not shown).

We also compared variabilities in 2 specific subregions of U3, nucleotides 19 to 373 (corresponding to the beginning of U3) and nucleotides 374 to 508 (the "regulatory" subregion) (Fig. 2A and B). Interestingly, HIV-2 group B viruses exhibited increased genetic variability in this regulatory subregion (19.0% versus 10.5% in group A; $P < 1.10^{-4}$) (Fig. 1B).

Seven sequences (9%) were found to have undergone APOBEC-induced hypermutation, equally distributed between the two viral groups (3 viruses belonging to group A [11%] and 4 to group B [11%]) (Fig. 1A).

Assessment of HIV-2 total DNA. Overall, HIV-2 total DNA was above the limit of quantification (LOQ) in only 24 patients (36%), with a median of 2.04 $\log_{10}$ c/10^6 PBMC (IQR = 1.81 to 2.15) (Fig. 3). HIV-2 total DNA was detectable below the LOQ in 40 patients (61%) and was undetectable for the remaining 2 patients (3%). The proportion of patients with a quantifiable reservoir was significantly higher in patients infected with HIV-2 group A than in those with group B (67% and 16%, respectively; $P < 0.001$) (Table 1). Among patients with a quantifiable cellular reservoir, HIV-2 total-DNA levels were significantly higher in group A-infected patients than in group B-infected patients (2.11 $\log_{10}$ c/10^6 PBMC [IQR = 1.94 to 2.16] versus 1.75 $\log_{10}$ c/10^6 PBMC [IQR = 1.68 to 1.81]; $P = 0.03$) (Fig. 3A).

When patients were stratified based on the CD4 cell count, patients with CD4 cell counts below 500/mm^3 more frequently had a quantifiable cellular reservoir than those with CD4 cell counts above 500/mm^3 (54% versus 32%) (Fig. 3C), although the difference was not statistically significant ($P = 0.20$). Nevertheless, when patients with a CD4 cell count below 500/mm^3 had a quantifiable reservoir, it was significantly larger than those for patients with CD4 cell counts above 500/mm^3 (2.17 $\log_{10}$ c/10^6 PBMC [IQR = 2.11 to 2.44] versus 1.94 $\log_{10}$ c/10^6 PBMC [IQR = 1.78 to 2.12]; $P = 0.02$) (Fig. 3D).

Association between mutations in LTR sequences and HIV-2 total DNA level. Mutations within the LTR were frequently observed, especially in the regulatory subregion in group B viruses. Sequences and *in silico* analyses revealed that PuB2, peri-κB,

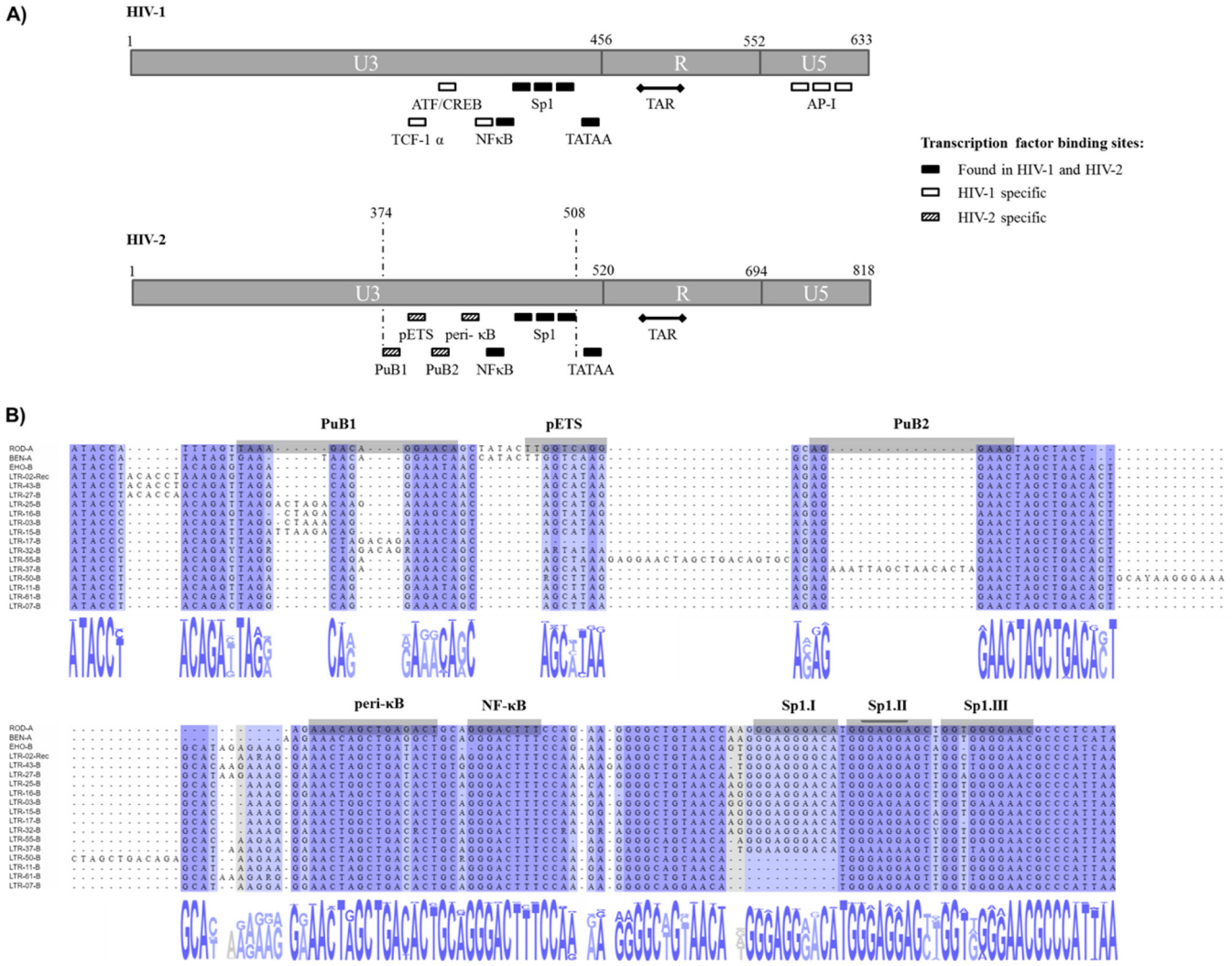

FIG 2 Schematic representation of the HIV-2 LTR and alignment of the regulatory subregions of selected HIV-2 group B sequences. (A) Representation of HIV-1 and HIV-2 LTRs, with the main TF binding sites depicted as boxes. The regulatory subregion corresponds to nucleotides 374 to 508 of the HIV-2 ROD reference strain. (B) Alignment of the regulatory subregion (nucleotides 374 to 508 of the ROD reference strain). The alignment was composed using sequences from the 2 HIV-2 reference strains (ROD and EHO for groups A and B, respectively) and from the 15 proviruses displaying insertions in this regulatory subregion (14 group B and 1 A/B recombinant). At each position, the intensity of shading is positively correlated with the degree of conservation among sequences. Known cellular TF binding sites described in ROD are highlighted in gray, and binding sites of Sp1 are labeled from Sp1.I to Sp1.II.

Sp1, and TATAA box binding sites were conserved (Fig. 1A). Interestingly, a short deletion in the region encompassing PuB1 and pETS binding sites was observed in all group B LTR sequences (Fig. 2). Those sequences conserved the core motif of the PuB1 binding site (AGGAA), and *in silico* analyses confirmed that the PuB1 binding site was still present. However, the deletion caused the loss of the pETS binding site (Fig. 2). All group B viruses also presented an insertion between the PuB2 and peri-κB binding sites (Fig. 2). The significance could not be determined through *in silico* analyses.

Fourteen group B viruses out of 38 (37%), as well as the A/B recombinant virus, presented at least one other insertion or deletion in the regulatory region: deletion of the first Sp1 binding site ($n = 4$), insertion in the PuB1 binding site ($n = 6$), or insertion at another location in the regulatory region ($n = 6$) (Fig. 2). These mutations were uncommon among group A viruses (2/27; 7%). Almost all the insertions and deletions were unique to one proviral sequence, and no statistical association was found between certain mutations and HIV-2 cellular reservoir levels (Fig. 3B). The only mutation that was repeatedly observed was the complete deletion of the first Sp1 binding site (Sp1.I) in 4 group B viruses ($n = 4/38$; 11%) (Fig. 2). Although the four patients

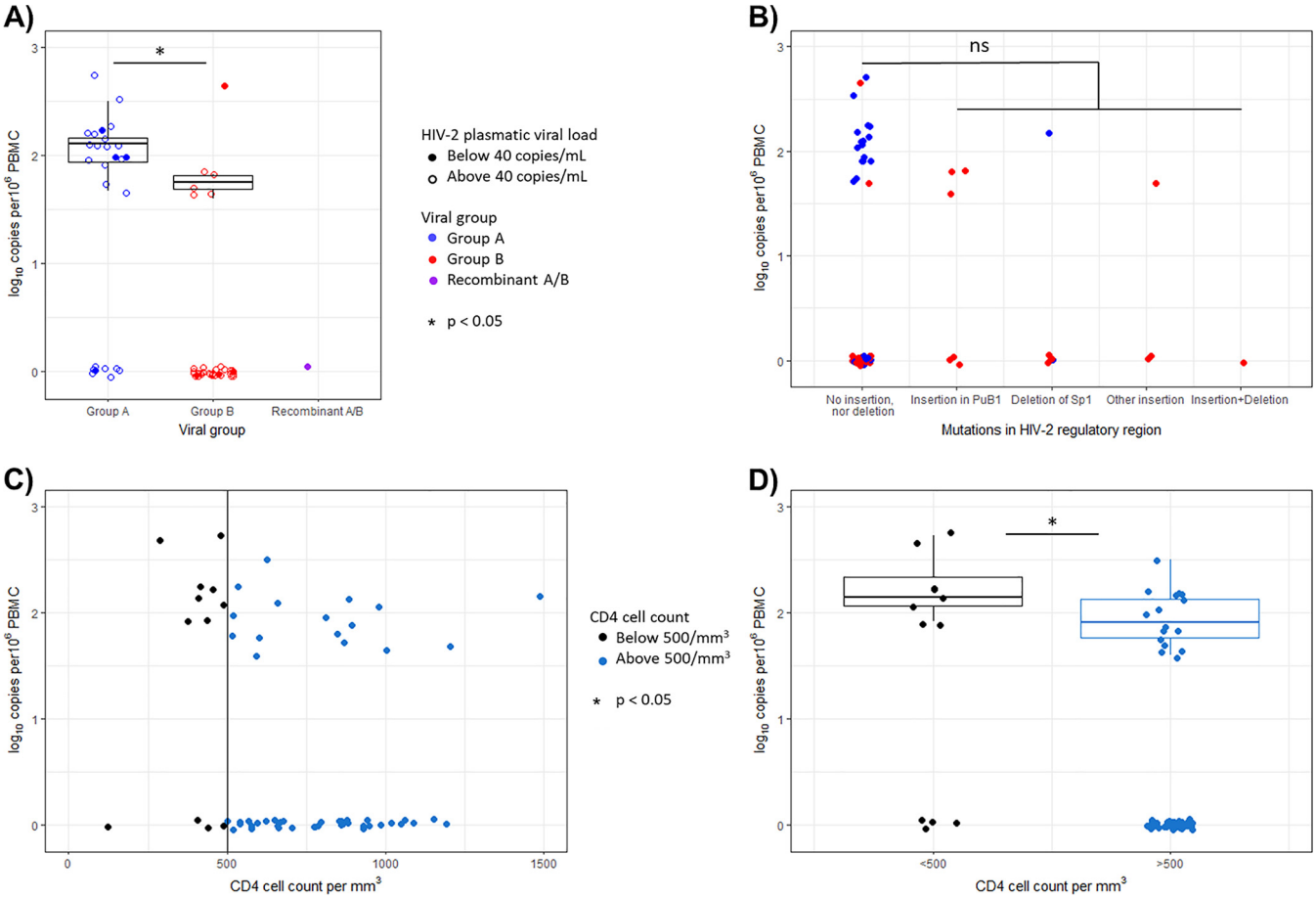

FIG 3 HIV-2 cellular reservoir quantification. (A) Quantification of HIV-2 total DNA. The HIV-2 cellular reservoir was quantified by an in-house technique. HIV-2 reservoir sizes are plotted separately for each viral group, in blue for group A, red for group B, and purple for the A/B recombinant. The box plots represent the median and interquartile ranges of quantifiable HIV-2 cellular reservoirs. *P* values were calculated with a Mann-Whitney test. (B) Comparison of HIV-2 cellular reservoirs based on the presence of insertions and/or deletions in the regulatory region. The insertions and/or deletions were observed in 17 viruses (14 group A, 2 group B, and 1 A/B recombinant). ns, not significant. (C) HIV-2 reservoir size based on CD4 cell count. The HIV-2 total DNA level of each patient is expressed as a function of the CD4 cell count. (D) Comparison of HIV-2 total-DNA levels between patients with CD4 cell counts below or above 500 CD4 cells/mm^3. The box plots represent the medians and interquartile ranges of quantifiable HIV-2 cellular reservoirs. HIV-2 cellular reservoir levels were compared using a Mann-Whitney test. (A to D) An arbitrary level of 1 copy per 10^6 PBMC was assigned when HIV-2 total DNA was quantifiable but below the limit of quantification (i.e., 6 copies per PCR), and the two patients (1 group A and 1 group B) with undetectable HIV-2 total DNA levels were not included.

presented small reservoirs (1 with 1.67 $\log_{10}$ c/10^6 PBMC, 2 with an HIV-2 DNA level below the LOQ, and 1 with undetectable HIV-2 DNA), the difference was not statistically significant (Fig. 3B).

Transcriptional activities of the HIV-2 LTR. We compared transcriptional activities, using plasmids derived from two HIV-2 strains, ROD and EHO, the reference strains for groups A and B, respectively. Different plasmids were constructed: (i) a ROD-LTR plasmid; (ii) EHO-LTR; (iii) ΔSp1-LTR, corresponding to the LTR of EHO lacking an Sp1.I binding site; and (iv) Rec-LTR, which corresponds to the A/B recombinant LTR identified (Fig. 4). We also constructed VAR-LTR, a chimeric LTR composed of ROD-LTR, except for the PuB1/pETS subregion, which was replaced by the shorter corresponding sequence of EHO-LTR lacking a pETS binding site (Fig. 4A).

The plasmids were transfected into HEK293T cells to assess the basal transcriptional activity of the promoters. The basal transcriptional activities of EHO-LTR and ΔSp1-LTR were halved compared to ROD-LTR ($P = 0.02$ for both), whereas that of Rec-LTR was 2-fold higher than that of ROD-LTR ($P < 0.001$) (Fig. 4B). Cotransfection with increasing amounts of HIV-2 Tat plasmid (pTat) showed a dose-response effect of LTR transactivation by Tat (Fig. 4B). When cotransfected with a high dose of Tat-encoding plasmid (5 ng), the transcriptional activity of ΔSp1-LTR remained 2-fold lower than those of

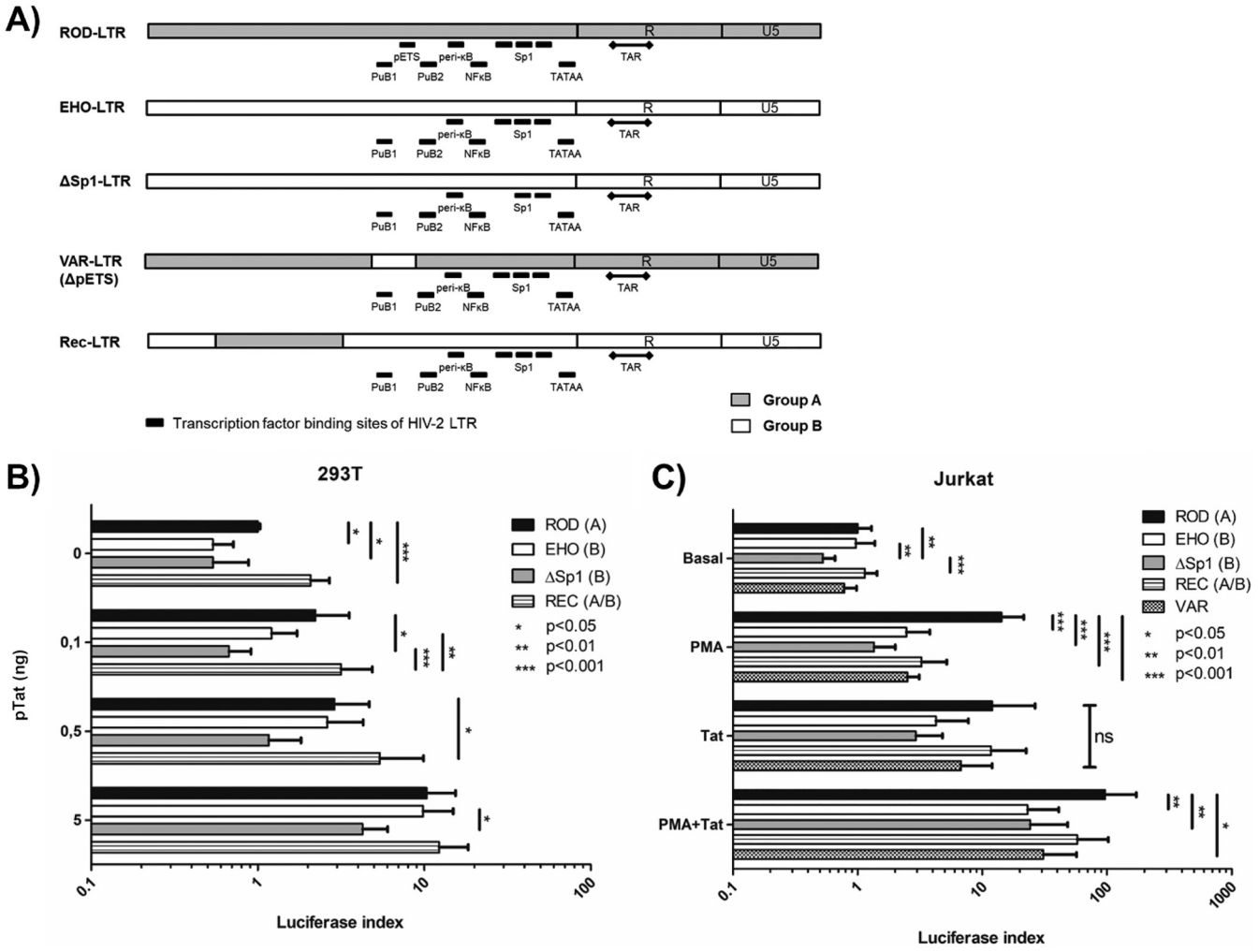

FIG 4 Transcriptional activities of HIV-2 LTRs. (A) Representation of the 5 different pLTR-Luc plasmids used in luciferase assays. ROD, complete LTR sequence of the HIV-2 group A strain ROD; EHO, LTR of the HIV-2 group B strain EHO; ΔSp1, LTR of EHO with deletion of Sp1.I, as identified in 4 patients; Rec, complete LTR sequence of the identified A/B recombinant; VAR, chimeric LTR constructed by replacing the PuB1/pETS subregion in the ROD sequence with the corresponding sequence of EHO lacking the pETS binding site. (B) Luciferase index in HEK293T cells transfected by pLTR-Luc and various amounts of pTat (0, 0.1, 0.5, and 5 ng). Luciferase indexes were obtained by dividing luciferase activities by the mean basal luciferase activity of ROD-LTR for each experiment. (C) Luciferase index in Jurkat cell clone 20 cells transfected by pLTR-Luc alone or activated by PMA and/or Tat. For Jurkat cell experiments, luciferase indexes were obtained by dividing the luciferase activity by the value of the MTT assay and then dividing by the value of the basal activity of ROD-LTR for each experiment. (B and C) Results (means and standard deviations) obtained through at least 3 independent experiments performed in triplicate. For each condition, the means were compared with a one-way ANOVA, and *P* values were calculated with a *post hoc* Tukey test. pLTR-Luc, plasmid containing the entire HIV-2 LTR which drives firefly luciferase expression; pTat, plasmid coding for HIV-2 Tat.

ROD-LTR and EHO-LTR ($P = 0.02$). Under the same conditions, the transcriptional activities of EHO-LTR and Rec-LTR were not significantly different from that of ROD-LTR (Fig. 4B).

Transient-transfection experiments were also performed on Jurkat cells to assess both basal transcriptional activity and transcriptional activity after cellular activation and/or Tat transactivation. The basal transcriptional activity of ΔSp1-LTR was 2-fold lower than that of ROD-LTR ($P < 0.01$), while EHO-LTR, Rec-LTR, and VAR-LTR had basal transcriptional activities similar to that of ROD-LTR ($P = 1$, 0.79, and 0.43, respectively) (Fig. 4C). LTR transcriptional activity in Jurkat cells was also positively correlated with the quantity of pTat transfected (data not shown). Cotransfection with 5 ng of pTat transactivated all LTRs by 3- to 10-fold (Fig. 4C). The increases of transcriptional activities after Tat transactivation were similar to those observed in HEK293T cells for all the plasmids (Fig. 4B and C). As observed in HEK293T cells, ΔSp1-LTR exhibited the

lowest Tat-mediated transcriptional activity, but the difference was not significant in Jurkat cells.

After cellular activation by phorbol myristate acetate (PMA), transcriptional activities were enhanced for all the LTRs, and ROD-LTR exhibited significantly higher activity than all the other LTRs: 10-fold higher than ΔSp1-LTR and 4- to 5-fold more than EHO-LTR, Rec-LTR, and VAR-LTR ($P < 0.001$) (Fig. 4C). No significant difference in the response to cellular activation was observed between EHO-LTR, ΔSp1-LTR, Rec-LTR, and VAR-LTR.

Transcriptional activities were also determined in Jurkat cells cotransfected with pTat and then activated by PMA (Fig. 4C). Under these conditions, the transcriptional activity of ROD-LTR was multiplied by a factor of 97 and that of Rec-LTR by a factor 49 ($P = 0.14$), while the transcriptional activities of EHO-LTR, ΔSp1-LTR, and VAR-LTR were enhanced only by a factor of 23 to 30 (Fig. 4C).

DISCUSSION

This is the first analysis of a large number of HIV-2 LTR proviral sequences obtained from ARV-naive patients and including viruses belonging to both HIV-2 epidemic groups. Until now, all studies have investigated only the HIV-2 group A LTR (19, 21, 24). We observed that the HIV-2 group B LTR displayed higher variability and a lower response to cellular activation.

In our study, the distribution of patients between groups A and B differs from what was previously observed in France (31). In 2001, 30% of patients were infected with HIV-2 group B in 2001 compared to 58% in the present study. However, this increase in the prevalence of group B viruses is consistent with the findings of other phylogenetic studies carried out in the French ANRS HIV-2 CO5 Cohort and with the birthplaces of enrolled patients (34). Most of them were born in West African countries known to have a high prevalence of HIV-2 group B infections (i.e., Mali and Ivory Coast) (4). In this study, only a small proportion of patients (36%) had a quantifiable HIV-2 total DNA level. This differs from observations of previous studies (32, 35), but the populations are distinct in terms of (i) CD4 cell counts, (ii) percentages of patients with a detectable plasma VL, and (iii) therapeutic status, thus hampering comparisons. Damond et al. reported that 79% of patients had a detectable HIV-2 total DNA level, but the patients were at more advanced stages of the disease: 55% of them had plasma VL above 100 c/ml (versus only 4.5% in our study), and 86% of them had CD4 levels under 500/mm^3 (versus 20% in our study) (35). Furthermore, half of them had received antiretroviral therapy (35). Similarly, Bertine et al. reported that 88% of patients had quantifiable reservoirs, but they were mainly infected with group A viruses (61% versus 41%) and frequently had a plasma VL above 100 c/ml (26% versus 4.5%) and a lower CD4 cell count (median, 521 versus 754/mm^3) (32). Interestingly, in our study, the proportion of quantifiable cellular reservoirs varied between the two groups: 67% of HIV-2 group A-infected patients versus 16% of HIV-2 group B-infected patients. As in previous studies (15, 33, 35), an inverse association between the HIV-2 total-DNA level and the CD4 cell count was found when patients were stratified according to their CD4 cell counts: below or above 500 CD4 cells/mm^3.

Regarding HIV-2 LTR variability, we reported high variability in the regulatory subregion encompassing most of the known TF binding sites, as has been described in HIV-1 (36). Our study provides insights into group-specific differences in the HIV-2 LTR. LTR genetic variability was greater in group B than in group A viruses, partly due to the numerous short deletions and insertions in group B sequences. Variability among HIV-2 group A LTR sequences was previously reported (37). Berry et al. reported that some patients infected by HIV-2 group A viruses harbored deletions or insertions in proviral DNA sequences and that the PuB1-pETS subregion may serve to discriminate between HIV-2 group A and B sequences (37). However, they did not investigate the consequences of this intergroup difference (37). We observed that PuB2, peri-κB, NF-κB, and Sp1 binding sites were conserved among LTR sequences, with few group-specific variations, as described in different HIV-1 subtypes (28, 38). The PuB1 binding site was

still identified by *in silico* analyses, while the pETS binding site was not present in any group B sequences.

A complete deletion of the first Sp1 binding site was observed in 11% of group B sequences, but it was not significantly linked to a change in the cellular reservoir level. Other insertions/deletions were observed within the LTR region, but they were patient specific. Thus, we could have lacked the statistical power to prove their impact on the level of the cellular reservoir.

To determine the impact of genetic variability in specific HIV-2 LTR subregions on transcriptional activity, we characterized the *in vitro* transcriptional activities of five LTRs of interest in HEK293T and Jurkat cells. The HEK293T cells were transfected to assess basal transcriptional activity and response to Tat transactivation, while the Jurkat cells enabled us to determine the impact of cellular activation on LTR transcriptional activity.

At the basal level, in Jurkat cells, ΔSp1-LTR expressed transcriptional activity reduced by half compared to EHO-LTR. Previous work has suggested that the widely expressed transcription factor Sp1 may be responsible for the basal transcriptional activity of HIV LTRs (39–41). Our findings in Jurkat cells are consistent with this hypothesis, as the loss of one Sp1 binding site impacts basal transcriptional activity. However, in HEK293T cells, EHO-LTR and ΔSp1-LTR exhibited similar basal transcriptional activity. This may be explained by differences in the level of expression of Sp1, or in other cellular TFs, between the two cell lines. When transactivated by pTat, all the LTRs displayed similar levels of transcriptional activity, except ΔSp1-LTR, whose activity was 2-fold lower than that of ROD-LTR in both HEK293T and Jurkat cells. However, the difference was significant only in HEK293T cells. In this cell line, ΔSp1-LTR was also significantly less transactivated than EHO-LTR when cells were cotransfected by a high dose of pTat. These results favor a limited impact of the loss of the first Sp1 binding site on the intensity of Tat-mediated LTR transactivation. This is consistent with the mechanism of LTR transactivation by Tat. It has been previously shown that, in addition to binding to the Tat-activated region (TAR) (42), Tat requires the presence of 3 intact Sp1 binding sites to efficiently transactivate the HIV-2 LTR (27).

When activation of Jurkat cells was induced by PMA, which activates T cells by its action on protein kinase C, transcriptional activities of all LTRs were increased, but at diverse levels. Transcriptional activity post-cellular activation was higher for ROD-LTR than for all the other LTRs. As the sole difference between ROD-LTR and VAR-LTR is the replacement of the PuB1/pETS subregion, this indicates the role of the subregion, and, notably, the loss of the pETS binding site, in the decreased response to cellular activation of group B LTRs in activated Jurkat cells. This is consistent with previous studies that reported that activated T cells, including Jurkat cells, expressed the Elf-1 protein, the only cellular TF known to bind to PuB1, PuB2, and pETS (26, 43). Moreover, it has been reported that the deletion of a single binding site—PuB1, PuB2, pETS, or NF-κB—is sufficient to severely impair the response to cellular activation (19). The A/B recombinant also lacked the pETS binding site, and it also exhibited a reduced response to cellular activation.

PMA and Tat exert an additive effect on LTR transactivation in Jurkat cells. The transcriptional activities of the five promoters were increased by 20- to 100-fold when activated by both PMA and Tat. However, cotransfection with pTat was not sufficient to overcome the difference observed between group A and group B LTRs after cellular activation. This limited response of group B LTRs to cellular activation may block or limit the switch from an early phase to a productive phase of infection (44, 45). This may partly explain the smaller size of the HIV-2 cellular reservoir observed in patients infected by HIV-2 group B in our study, as lower transactivation could lead to a lower number of newly infected cells and thus limit the expansion of the cellular reservoir.

Differences in LTR responses to activation have also been described for HIV-1 subtypes, such as an increased response to cellular activation of B′ and C subtypes compared to the recombinant form CRF01_AE (28, 29). Qu and colleagues reported that subtype-specific genetic diversity in the subregion encompassing TF binding sites in the HIV-1 LTR (nucleotides −138 to 0) can have a large impact on their transcriptional activities (29).

This study has some limitations. LTR transcriptional activities were determined in two different cellular models, HEK293T and Jurkat cells, but not in primary cells that may express TFs at different levels (25, 46). Moreover, although differences were observed in transcriptional activities between HIV-2 group A and B viruses, patients differed only in their proviral loads. Plasma VL and CD4 cell counts were similar for both groups. In HIV-1, most studies have failed to link disease progression to specific mutations within the LTR. Since mutations in the HIV-2 LTR do not translate into differences in clinical outcomes, we can hypothesize that other mutations in the retroviral genome could either compensate for the lower transcriptional activity or alter viral replication. Only *in vitro* experiments evaluating viral replication, the level of the cellular reservoir, and the reactivation of molecular clones harboring specific mutations could decipher the real impact of each mutation observed.

We observed 3 main differences between HIV-2 groups A and B: (i) greater diversity within the regulatory subregion in group B sequences, (ii) a smaller cellular reservoir in patients infected with HIV-2 group B, and (iii) reduced PMA-activated transcriptional activity of the group B LTR. Sequence analyses and transfection experiments highlighted the importance of pETS binding sites in the response of the HIV-2 group A LTR to cellular activation. Furthermore, the deletion of one of the Sp1 binding sites impaired either basal or Tat-mediated transcriptional activities, depending on the cell line. However, this difference was moderate and was no longer observed when transfected cells were activated by both PMA and Tat, a model close to *in vivo*-infected cells, in which transactivation by Tat occurs after cellular activation. Therefore, this mutation might not be as clinically important as the deletion of the pETS binding site.

To our knowledge, this is the first study to reveal notable differences in LTR transactivation between HIV-2 group A and group B. Our results suggest that reactivation of latent proviruses may be impaired in patients infected with an HIV-2 group B strain lacking the pETS binding site. These results should be verified by further *in vitro* experiments, especially reactivation of LTR-mutated HIV-2 proviruses.

MATERIALS AND METHODS

Patients. All antiretroviral-naive patients included in the French ANRS HIV-2 CO5 Cohort from whom whole-blood samples were obtained between January 2015 and February 2016 were included in this study. Plasma and PBMC were collected using Ficoll lymphocyte separation medium (Eurobio, Courtaboeuf, France) and stored at −80°C until DNA extraction.

HIV-2 LTR sequencing. DNA was extracted from PBMC using a QIA Symphony SP DNA minikit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The HIV-2 3′ LTR region was amplified in a two-step PCR. The PCR primers used were as follows: 3LTR-F1 (5′-GGGCTATAGGCCWGT WTTCTCYTCCCC-3′; melting temperature [T_m] = 68.8°C), 3LTR-R1 (5′-AAGGGTCCTAACAGACCAGG-3′; T_m = 59.4°C), 3LTR-F2 (5′-GGGGACTGGAAGGGMTGTWTTAYA-3′; T_m = 62.7°C), and 3LTR-R2 (5′-GACCAG GCGGCGACTAGG-3′; T_m = 62,8°C). Primer 3LTR-F1 was first described by Machuca et al. (47). The nested-PCR product encompassed almost the whole LTR region (nucleotides 9499 to 10275 of the reference strain BEN, GenBank accession number M30502), lacking only the last 60 nucleotides of U5, which are highly conserved in HIV-2. First-round PCR was performed with 10 μl of extracted DNA and 40 μl of a mixture of 5 μl of buffer, 5 μl of $MgCl_2$, 0.2 μl of a mixture of deoxynucleotide triphosphates (dNTPs) at 2.5 mM, 2 μl of 3LTR-F1 and 4 μl of 3LTR-R1 primers at 10 μM, 0.25 μl of *Taq* Gold polymerase (Thermofisher, Applied Biosystems, Foster City, CA, USA), and 24 μl of DNase-free water. The following PCR program was used: 12 min at 94°C, then 40 amplification cycles (94°C for 30 s, 50°C for 30 s, and 72°C for 2 min), and finally 72°C for 7 min. Nested PCR was performed on 5 μl of the first-round PCR products diluted in 5 μl of DNase-free water and added to the same mixture described above, except 2 μl of 3LTR-F2 and 4 μl of 3LTR-R2 primers at 10 μM replaced primers 3LTR-F1 and 3LTR-R1. The same amplification program was used, except the hybridization temperature was set to 55°C. The nested-PCR products were analyzed with a capillary electrophoresis automat, Caliper Labchip GX (Perkin Elmer, Waltham, MA, USA), to verify amplification. Sequencing reactions were run using an ABI Prism BigDye Terminator kit with the 3LTR-F2 and 3LTR-R2 primers on an automated sequencer (ABI Prism 3130 XL; Applied Biosystems). LTR sequences were analyzed using Geneious v9.0 (Brisbane, Australia) and aligned using both ClustalW and manual editing.

HIV-2 total-DNA quantification. Real-time PCR of the human albumin gene (48) was performed on total DNA extracted from PBMC to determine the number of cells per sample, as previously described (44). Quantification of total HIV-2 DNA was performed using an in-house real-time PCR assay with a threshold of 6 copies per PCR. Samples with a signal below this LOQ were defined as detectable. HIV-2 total DNA in PBMC was expressed in copies of HIV-2 DNA per 10^6 PBMC. The assay was developed by the ANRS AC11 Quantification Group (49).

HIV-2 RNA quantification. One milliliter of supernatant was extracted using MagNAPure with an LC total nucleic acid isolation kit (Roche). The HIV-2 RNA plasma VL was determined using a Biocentric HIV-2 RNA kit (Biocentric, Bandol, France), with a limit of quantification of 40 c/ml (50).

Bioinformatic analysis. (i) Phylogeny and recombination. Recombination within the LTR region was checked using RDP4 vBeta 4.97 software (51). The A/B recombinant identified by RDP4 was excluded from further phylogenetic analyses, as recommended (52). Using 3 reference strains (A.SN.85.ROD.M15390 [ROD] and A.DE.x.BEN.M30502 [BEN] for subtypes A1 and A2, respectively, and B.CI.x.EHO.U27200 [EHO] for group B) and all LTR sequences, a consensus phylogenetic tree was built by the maximum-likelihood method using PhyML software v3.1 under a general time-reversible model with a gamma parameter at 4 (53). The robustness of the tree was assessed by bootstrapping with 1,000 replicates. The tree was edited with FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

(ii) Genetic-distance calculations. Paired genetic distances were calculated using HyPhy v2.2.4 (54) by computing the pairwise distance between LTR sequences and their group references (ROD for HIV-2 group A and EHO for HIV-2 group B). Genetic distances were expressed as percentages of substitutions per nucleotide. Sequences corresponding to primers were deleted from LTR sequences in order not to misrepresent genetic variability.

(iii) Hypermutation analysis. Hypermutation was assessed with Hypermut2.0 software (55), available online (https://www.hiv.lanl.gov/content/sequence/HYPERMUT/hypermut.html). Hypermut2.0 detects hypermutation induced by the APOBEC3G or APOBEC3F protein (56, 57). We used prototypic strains ROD and EHO as references. LTR sequences with a P value of <0.05 were considered APOBEC hypermutated.

(iv) Identification of TF binding sites. LTR sequences were analyzed with MatInspector v8.3 (Genomatix, Munich, Germany) and PROMO v3.0.2 in order to verify the presence of known TF binding sites and to identify putative TF binding sites (58, 59). Only binding sites of TFs belonging to protein families known to bind to the HIV-1 LTR were included in our analyses (60, 61).

Cell lines. HEK293T (ATCC no. CRL-3216) and Jurkat clone 20 (62) cell lines were provided by the Virus and Immunity Unit, Pasteur Institute, Paris, France. HEK293T cells were cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Jurkat cells were cultured in RPMI medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin.

LTR transcriptional activity. The transcriptional activities of LTR regions were determined by transfecting cell lines with plasmids carrying the *Photinus pyralis* luciferase gene driven by HIV-2 LTR (pLTR-Luc). The expression of luciferase was correlated with the promoter's activity, i.e., with LTR transcriptional activity, and was enhanced by cotransfection of an HIV-2 Tat plasmid (pTat) and/or cellular activation by PMA (Biontex, Munich, Germany). HIV-2 Tat and HIV-2 LTR sequences were synthetized by Eurofins Genomics (Ebersberg, Germany) and then subcloned in pcDNA3 and pGL4.14[luc2/Hygro] vectors (Promega, Madison, WI, USA), respectively.

HEK293T cells were transfected using Metafectene (Biontex, Munich, Germany). For each well, 50 ng of plasmid DNA was mixed in 4 μl of Metafectene and 46 μl of phosphate-buffered saline (PBS) and then incubated for 15 min at room temperature. The mixture was added to 5×10^5 cells, thoroughly mixed, and then incubated for 15 min at room temperature. The transfected cells were diluted with DMEM, and 5×10^4 cells were seeded per well in triplicate under all conditions. After 20 h of culture at 37°C with 5% CO_2, 50 μl of Bright Glo luciferase (Promega, Madison, WI, USA) was added to 50 μl of cells. After 150 s, complete cell lysis was achieved, and luciferase activity was assessed using a luminometer. The luciferase index was calculated by dividing the median luciferase activity under each condition by the median luciferase activity for ROD-LTR at the basal level.

Transcriptional activities were also determined in transfected Jurkat clone 20 cells, using a DEAE-dextran method adapted from a previous publication (63). Briefly, 2×10^5 Jurkat cells were seeded per well, washed, and then mixed with 15 μl of DEAE-dextran-DNA containing 55 ng of plasmid DNAs (50 ng of pLTR-Luc and 5 ng of pTat or empty pcDNA3) for 20 min at room temperature. The transfected cells were then diluted with 225 μl of RPMI 1640 medium supplemented with 2% glutamine, 5% fetal calf serum (FCS) (ThermoFisher, Gibco, Waltham, MA, USA), and antibiotics (penicillin and streptomycin) and incubated for 1 h at 37°C with 5% CO_2. The cells were washed and then suspended with 250 μl of RPMI 1640 medium containing 10% FCS and antibiotics. After 22 h at 37°C with 5% CO_2, the cells were centrifuged, the supernatant was discarded, and the pellet was suspended with 200 μl of RPMI medium (alone or with 1 μl of a PMA solution in order to obtain a final PMA concentration of 100 ng/ml). After 8 h of incubation, the 96-well plate was centrifuged, and 150 μl of medium was removed from each well. A luciferase assay was performed on the remaining 50 μl by adding 50 μl of Bright Glo luciferase. Simultaneously, an MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay was carried out to verify cell viability (63). Luciferase activities and MTT values were measured with a luminometer. In order to assess LTR transcriptional activity, data were normalized with the MTT value and then divided by the ROD-LTR basal activity to obtain a luciferase index.

Three to five independent transfection experiments were performed in triplicate under each condition in both cell lines.

Statistics. One-way analysis of variance (ANOVA) and *post hoc* Tukey, Mann-Whitney, and Fisher's exact tests were performed using R v3.2.4 (http://www.R-project.org), with the significance level defined as a P value of <0.05.

Ethics statement. The French ANRS CO5 HIV-2 Cohort has been approved according to the French ethic laws. The project has been approved by the local institutional review board CCP IDF XI (Comité de Protection des Personnes, Ile-de-France) on 24 January 2002. Collection and transmission of patients'

data was approved by the CCTIRS (Comité Consultatif sur le Traitement de l'Information en matière de Recherche dans le Domaine de la Santé) on 14 November 2002 and by the CNIL (Commission Nationale de l'Informatique et des Libertés) on 30 December 2002. Written informed consent was obtained from all patients at the time of inclusion in the cohort.

Data availability. The data related to this study, including nucleotide sequences, are available in a public repository (https://doi.org/10.17605/OSF.IO/XFUZR).

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